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Antibacterial Activity and Protein Sequences of Actinomycetes Isolated from Coastal Area of Niger Delta against Human and Fish Pathogens

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ABSTRACT:
Actinomycetes are known to produce potential secondary metabolites which possess biological activity. This knowledge spurred the present study in which the antibacterial activity and protein sequences of Actinomycetes isolated from Coastal Area of Niger Delta against human and fish pathogens were evaluated. Marine sediment and water samples were collected from ten (10) different points from Onne light flow terminal seaport located in Eleme Local Government Area of Rivers State, Nigeria. A total of fifteen (15) Actinomycetes were isolated on Modified Glycerol Starch Casein Nitrate Agar using spread plate technique. All the isolates were characterized and identified using standard procedures. The API system of identification used revealed that thirteen of the isolates belong to the genus *Streptomyces* while two belong to the genus *Actinomyces*. The isolated marine Actinomycetes were screened for their antibacterial activities against four human bacterial pathogens namely:- *Staphylococcus aureus, Escherichia coli, Salmonella Typhi* and *Shigella dysenteriae* and four fish bacterial pathogens:- *Staphylococcus aureus* P1, *Vibrio* sp. MF2, *Salmonella* sp. and *Vibrio* sp. S1. The antibacterial screening revealed that amongst the fifteen (15) isolates, ten (10) showed inhibitory activities against the human pathogens while fourteen (14) strains showed inhibitory activities against the fish pathogens. The two most potent isolates were ATS-3 and ATS-1 which were both identified as *Steptomyces lavendulae*. ATS-3 showed the highest inhibitory activity against *S. dysenteriae* (30.00 ± 0.25 mm) and lowest activity against *S. aureus* (10.00 ± 0.16 mm) while ATS-1 strain showed highest inhibitory activity against *Vibro* sp. S1 (25.00 ± 5.20 mm) and lowest inhibitory activity against *S. aureus* P1 (11.00 ± 2.24 mm). Their inhibitory activities were statistically significant with P values less than 0.05 ($P<0.05$). The protein profiling of the partially purified whole cell proteins of ATS-3 and ATS-1 on SDS-PAGE exhibited apparent lower and upper molecular weights of 71.7 kDa and 47.5 kDa and 70.4 kDa and 41.16 kDa respectively. The result of the taxonomic correctional analysis revealed that the protein sequences of the two strains were closely related to each other with 80 % similarities suggesting that the strains belonged to the genus *Streptomyces*. Thus, the fact that majority of Actinomycetes strains (66.67 %) in the studied area produced potential bioactive compounds against the human and fish bacterial pathogens studied revealed that the marine actinomcycetes from the Onne light flow terminal seaport could serve as potential sources of novel antibiotics.
Keywords: Actinomycetes, Antibacterial activity, Fish pathogens, Human pathogens, Coastal area, Niger Delta.

INTRODUCTION:

Actinomycetes are Gram-positive bacteria with high G+C content. Actinomycetes play an important role in recycling wastes in the environment and they are also the producers of thousands of metabolic products which exhibit biological activity. After the discovery of the broad spectrum antibiotic Streptomycin by Waksman and Schatz, more attention was paid towards the Actinomycetes for isolation of many more antibiotics [1].

As the frequency of novel bioactive compounds discovered from terrestrial Actinomycetes decreases with time, much attention has been focused on screening of Actinomycetes from diverse environments for their ability to produce new secondary metabolites. Studies have shown that Actinomycetes isolated from the marine environment are metabolically active and have adapted to life in the sea. *Streptomyces* are especially prolific and can produce many antibiotics (around 80 % of the total antibiotic production) and active secondary metabolites [2].

More than 70 % of our planet's surface is covered by oceans and life on Earth originated from the sea. In some marine

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ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than that in the tropical rainforests. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine Actinomycetes have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds [3].

The most relevant reason for discovering novel secondary metabolites is to circumvent the problem of resistant pathogens, which are no longer susceptible to the currently used drugs. Marine Actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, insecticidal and enzyme inhibition [4]. There has been a steady increase in the number of species of bacteria implicated in fish diseases. The common fish pathogenic bacterial species belong to the genera *Vibrio, Aeromonas, Flavobacterium, Yersinia, Edwardsiella, Streptococcus, Lactococcus, Renibacterium* and *Mycobacterium* [5, 6]. The use of antibiotics to control human and fish diseases has met with limited success and has the potential danger of antibiotic resistance development in human and aquatic bacteria [7]. As aquaculture is one of the fastest growing food production industries in the world, demand for sustainable ways of combating fish diseases is gaining significance. There is tremendous scope for developing novel vaccines and therapeutic drugs against bacterial fish pathogens [8].

The Niger Delta region of Nigeria has a wide coastal area that is highly rich in biodiversity. The Niger Delta is located in the Atlantic coast of Southern Nigeria and is the world's second largest delta with a coastline of about 450 km which ends at Imo river entrance [9]. Onne Light Flow Terminal Seaport is situated on the Bonny River Estuary along Ogu Creek and account for over 65 % of the export cargo through the Nigerian Sea Port. Many reports describe the coastal area of India, China, America and Europe as major source of Actinomycetes [10]. However, only few reports are available on Actinomycetes diversity of marine water and sediment, which is a bountiful resource in Africa and most especially in the Niger Delta region of Nigeria. Furthermore, there are also dearth reports on the use of marine actinobacteria as antimicrobial agents against human and fish pathogens as well as protein sequences in Nigeria since the current treatment methods are ineffective and have many practical difficulties. These problems and gaps necessitated and justified the reason for the present study which aimed at evaluating the antibacterial activities and protein sequences of Actinomycetes isolated from Coastal Area of Niger Delta against human, fish and shell fish bacterial pathogens.

MATERIALS AND METHODS:

1. Study area

The study area was Onne Light Flow Terminal Seaport located in Eleme Local Government Area of Rivers State. Onne Light Flow Terminal had two terminals: light and heavy and the sampling site is about 35 km east from Port Harcourt capital city of Rivers State and 7 km from Onne town. These sites were geo-referenced using Handheld Global Positioning System (GPS) GPSMAP 76sc with the coordinates obtained from the sampling points or positions. Onne Light Flow Terminal Seaport was located between latitude 4°41'32.58"N and 4°41'58.18"N and longitude 7°9'26.34"E and 7°10'48.82"E with average elevation of 2.3 m respectively. Onne Light Flow Terminal Seaport is a port of Nigeria and the largest oil and gas free zone in the world supporting exploration and production for Nigerian activities. It is situated on the Bonny River Estuary along Ogu Creek and account for over 65 % of the export cargo through the Nigerian Sea Port.

2. Sample collection and processing

Ten samples were collected randomly at each designated point in the three particular sampling sites and mixed together after which a total of two representative sediment

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and water samples were taken for the analysis. All the samples were collected from the intertidal zone of the sampling sites during ebbtide based on routine survey. The surface aerobic sediment samples were collected with a 95 % ethanol- sanitized plastic spatula at 5 cm depth inside 95 % ethanol- sanitized wide mouthed plastic containers. The water samples were collected at the air-water interface by hand dipping the 95 % ethanol- sanitized cylindrical shaped 2 L plastic containers. The containers were rinsed with the water samples before collecting the samples. All the composite or representative sediment and water samples were placed into a sterile polythene bags in ice packed coolers to keep them under a temperature not more than 4°C and then transported to the Microbiology laboratory and stored at 4 ^{0}C in refrigerator [11, 12].

3. Isolation of Actinomycetes

Sample (about 10 g) of air-dried soil were mixed with sterile distilled water (100 ml). The sediment suspension were heated in water bath at 70 °C for 1 hr and then shaken at 200 rpm for 30 mins at 30 °C. One ml of soil mixture was transferred to 9 mls of sterile distilled water and subsequently to final dilution 10^{-3}. About 0.1 ml of final dilution was spread plated in Modified Glycerol Starch Casein Nitrate Agar supplemented with Cyclohexamide (100 µg /ml) and Nalidixic acid (25 µg /ml) to minimize fungal and bacterial contaminations respectively. Plates were incubated by inversion at room temperature (30 ± 2 °C) for 14 days. Following incubation, colonies (30 – 300) that were seen on the plates were carefully counted using a colony counter and results obtained were recorded [1, 13, 14].

4.0 Purification and maintenance of Actinomycetes

The isolated colonies with different morphology were streaked on sterile glycerol starch casein nitrate agar plates from plates with high number of colonies and incubated by inversion at room temperature (30 ± 2 °C) for 14 days. Pure Actinomycetes cultures were transferred to yeast - malt extract agar (ISP 2- Yeast extract 3 g, Malt extract 5 g, Glucose 5 g, Agar 20 g, Distilled water 1000 ml) and preserved at 4 ± 2 °C refrigeration temperature [15, 16].

5.0 Morphological characterization and identification of Actinomycetes isolates

5.1. Macroscopic identification

The isolated Actinomycetes were characterized by inoculation on four different International Streptomyces Project (ISP) media. The media are ISP 1 (HCl 3 ml, Casein 15 g, Yeast extract 3 g, Agar 20 g, Distilled water 1000 ml); ISP 2 (Yeast extract 3 g, Malt extract 5 g, Glucose 5 g, Agar 20 g, Distilled water 1000 ml); ISP 3 (Oatmeal 15 g, Agar 20 g, $FeSo_4.7H_20$ 1 g, $Mncl_2.4H_20$ 1 g, $ZnSo_4.7H_20$ 1 g, Distilled water 1000 ml) and ISP 6 (Beef extract 10 g, Peptone 3 g, Yeast extract 1 g, $FeNH_4$ Citrate 0.5 g, K_2PO_4 1 g, Sodium disulphate 0.1 g, Agar 20 g, Distilled water 1000 ml). Plates were incubated by inversion at room temperature (30 ± 2 °C) for 7 - 14 days. These were observed under high power magnifying lens. The colony morphology with respect to colour, aerial and substrate mycelia, size, branching and nature of colony were observed and identified [10, 13, 14, 16, 17].

5.2 Microscopic observation

As described by Arora [18] and Willey *et al.* [19], the isolates were Gram stained, endospore stained and acid fast stained. Following staining, the stained cells were viewed using microscope and the results were recorded.

5.3 Coverslip culture technique

According to Randasamy *et al.* [20], sterile modified glycerol starch casein nitrate agar was prepared and the respective cultures were swabbed on the medium and 2 - 3 sterile square cover slips were inserted at an angle 45 ° and incubated at room temperature (30 ± 2 °C) for 7 - 14 days. Following incubation, the cover slips were carefully removed and wet mount technique were performed and observed under high power magnification (X40). The morphological arrangement of the

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spores and spore – bearing structures were observed and identified.

5.4 Biochemical characterization of Actinomycetes

The following biochemical tests were carried out: Indole, Urea, Glucose, Mannitol, Lactose, Sucrose, Maltose, Salicin, Xylose, Arabinose, Gelatin, Esculin, Glycerol, Cellobiose, Mannose, Melezitose, Raffinose, Sorbitol, Rhamnose, Trehalose and Catalase. These tests were done following the instructions on the Analytical Profile Index (API) kit manual and as described by Cheesbrough [21]. The isolates were identified using API Identification System or software.

6. Source of human bacterial pathogens

The pure cultures of four human bacterial pathogens namely *Staphylococcus aureus, Escherichia coli, Salmonella Typhi* and *Shigella dysenteriae* were obtained from the Department of Pharmaceutical Microbiology and Biotechnology Laboratory, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu, Nigeria.

7. Source of fish and shell fish pathogens

The fish and shell fish products which include cat fish, mould fish, cray fish, periwinkle and snail were bought at Cemetery Market Aba, Abia State, Nigeria. The market is one of the largest in the city of Aba known for sales of food stuffs. The fish and shell fish products were preserved by sun drying aseptically using sterile containers. After drying, the samples were transferred to sterile capped round mouthed plastic containers and transported to laboratory for isolation of fish and shell fish bacterial pathogens.

8. Isolation of fish and shell fish pathogens

The pathogens were isolated from fish and shell fish products above. The samples were grinded aseptically and 1 g of the each sample was weighed and serially diluted with sterile distilled water up to 10^{-3} dilution. Zero point one ml of each serially diluted samples was spreaded on Mannitol Salt Agar (MSA), Thiosulfate Citrate Bile Salts Sucrose Agar (TCBSA) and *Salmonella Shigella* Agar (SSA). The plates were incubated by inversion for 24 hrs at 37 °C as described by Dharmaraj [22].

9. Biochemical characterization

The following biochemical tests specific to the bacterial pathogens were carried out: oxidase, citrate, coagulase and triple sugar iron tests. They were carried out to confirm the presence of *Vibrio* spp., *Staphylococcus aureus and Salmonella* or *Shigella* spp. respectively. The observations were compared with the manual described by Murray *et al.* [23]

10. Antimicrobial screening of the marine Actinomycetes isolates

10.1. Primary screening

10.1.1. Streak – plating techniques

The preliminary antibacterial activities of the 15 pure Actinomycetes isolates were tested by cross-streak plate method [14, 16, 24, 25, 26] against human, fish and shell fish bacterial pathogens mentioned above on sterilized Mueller Hinton agar (MHA). Each plate was streaked with one isolate at the center and incubated at room temperature (30 ± 2 °C) for 7 - 14 days. After observing a good ribbon like growth of the Actinomycetes, the bacterial pathogens were streaked at right angle (90 °) to the original streak of each Actinomycetes isolate and incubated at 30 ± 2 °C. The inhibition zone (mm) was measured after 24 and 48 hrs. Control plates were also maintained without inoculating Actinomycetes/bacteria to assess the normal growth of the pathogenic bacteria and Actinomycetes.

10.2. Secondary screening

10.2.1. Extraction of bioactive compound

The cultures of the two (2) most potent strains of the Actinomycetes from the primary screening were prepared as broth culture by inoculating the isolates into sterile Yeast – Malt Extract (YME) broth and incubated at room temperature (30 ± 2 °C) for 5 days. Then, 5 ml of the broth culture was transferred into submerged culture of 250 ml flask containing 50 ml of modified starch casein nitrate broth and incubated at room

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temperature (30 ± 2 ºC) for 7 - 14 days. The medium was shaked with rotary shaker at 150 rpm. After growth, the fermented broth was collected and centrifuged at 5000 rpm for 10 mins and then filtered through Whatman No. 1 filter paper. Antimicrobial compound containing supernatant was extracted using equal volume of solvent (V/V). The culture filtrates were extracted twice with organic chloroform solvent and the pooled solvent extracts were evaporated to dryness to yield a crude residue before using for antimicrobial assay [13, 14, 25].

10.2.2. Agar well diffusion assay

Test organisms (bacterial pathogens) mentioned in primary screening were also used in secondary screening test. The test organisms were first grown as 24 hrs broth culture at 37 ºC using nutrient broth. The cultures were swapped on Muller Hinton agar (MHA) medium. The five respective wells (6 mm in diameter) generated by the sterile stainless steel cork borer were loaded with 50 µl of crude extracts. The plates were incubated at 37 ºC for 24 hrs. Antimicrobial activity (secondary metabolic activity) was estimated by measuring the inhibition zone diameters. Zone of inhibition was measured by using metre rule scale. The activity of 50 µl of spent medium of the two Actinomycetes isolates was compared with the activity of standard antibiotics (100 µg/ml ciprofloxacin). The above tests were repeated 3 times in time and in space [14, 24, 26].

11. Determination of protein profile from Actinomycetes most potent strains

11.1. Protein profile

This was done to identify differentiation among the strains that exhibited bioactivity. The molecular weights and purity of the whole cell proteins of the two most potent strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 were determined using Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) method with slight modification as described by Laemmli [27], Dharmaraj [22] and Dhananjeyan *et al.* [28].

11.2. Preparation of bacterial whole cell protein

The isolates were grown in their respective broth medium at (30 ± 2 ºC) for 7 days. The cells were harvested by centrifugation at 8000 rpm for 1 min. The pellet was washed with 1 M Tris HCl buffer (pH = 6.80) and resuspended in 10 ml of the same buffer and vortexed. Then 80 ml of the sample buffer (1 M Tris HCl pH = 6.8, 10 % SDS, 10 % glycerol, 5 % b -mercaptoethanol and 0.003 % Bromophenol blue) and 6.5 ml of b-mercaptoethanol were added to the preparations and boiled immediately in water bath at 100 ºC for 5 mins. After boiling, the samples were placed on ice for 5 mins and centrifuged at 8000 rpm for 1 min.

11.3. Preparation of SDS-PAGE

For SDS-PAGE, 12.5 % separating gel and 6.5 % resolving gel were prepared. A volume of 30 µL of each sample was loaded on gel and was run on mini gel electrophoresis at 200 V, 0.006 A for 2 hrs and stained in a solution containing 0.1 % (mass per volume) Coomassie blue, 10 % (volume) acetic acid and 30 % (volume) methanol. Destaining was performed in a solution containing 10 % (volume) acetic acid and 35 % (volume) methanol. The molecular weights of the partially purified whole cell proteins were determined in comparison with standard molecular weight markers (molecular mass range: 14.4 – 116.3 kDa).

11.4. Taxonomic correctional analysis

The taxonomic correctional analyses were done using PermutMatrix software and evolutionary distance or clustering of the isolates were computed using nearest neighbour hierarchial cluster algorithmic method. The similarity between the proteins sequences of the two potent strains of Actinomycetes were represented in the cluster dendogram [29].

12. .Statistical analysis

The data were analyzed using Graph-Pad Prism statistical software version 7.00 (GraphPad Software Inc. San Diego,

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California). All values were expressed as mean ± standard deviation (S.D). One - way analyses of variance (ANOVA) and T-TEST were performed on the obtained data to determine the significant differences among the means of antibacterial activity of the Actinomycetes isolates and their controls followed by post Tukey's multiple comparison test. The differences were considered statistically significant at 95 % confidence intervals if the probability is less than 0.05 ($p < 0.05$) [10].

RESULTS:

1. Isolation of Actinomycetes

The distribution of Actinomycetes in marine water and sediments samples is presented in Table 1. From the result, marine water was found to be higher in microbial count (4.09 ± 0.05 logCFU /ml) than the marine sediment microbial count (3.85 ± 0.08 logCFU /ml). There was no significant count ($P > 0.05$) between sediment and water samples.

2. Characterization and identification of Actinomycetes isolates

The cultural characteristics of Actinomycetes isolates on ISP 1, ISP 2, ISP 3 and ISP 6 are presented in Table 2, 3, 4 and 5 respectively. From the results, most isolates were grayish green and yellowish green in aerial mycelium and pinkish and dark brown in substrate mycelium with melanoid pigment produced only by strains ATS-13 and ATS-15 on ISP 6. The biochemical and morphological characterization of marine Actinomycetes isolates is presented in Table 6. From the results, all isolates were positive to Gram staining, catalase, glucose, mannose, trehalose tests while negative to acid fast and endospore staining, mannitol, indole and esculine tests. The rest of the isolates had variable reactions to the other biochemical tests. All were cocci in shape except ATS 7 and 8. Most isolates were spiral followed by reticulum and rectiflexible in spore structural arrangements. The predominant genus belong to *Streptomyces* and the least genus belong to *Actinomyces* as presented in Table 7.

3. Isolation and characterization of fish and shell fish pathogens

The biochemical characterization of fish and shell fish bacterial pathogens is presented in Table 8. From the result, *Vibrio* sp. MF2 and *Vibrio* sp. S1 were positive to oxidase test, *Salmonella* sp. and *Vibrio* sp. were positive to citrate test, *S. aureus* P1 was positive to coagulase test while *Salmonella* sp. was positive to hydrogen sulphide production.

4. ***Antimicrobial screening***

The results of the antibacterial activities of the Actinomycetes isolates against human, fish and shell fish bacterial pathogens are shown in Figures 1 and 3 while the results of the secondary metabolic activities of the crude chloroform extract of *Streptomyces lavendulae* ATS-3 and *Streptomyces lavendulae* ATS-1 against human, fish and shell fish bacterial pathogens are shown in Figures 2 and 4 respectively. From the Figure 1 result, 6 strains (40.00 %) were active against *S. aureus,* 7 strains (46.67 %) were active against *E.coli,* 5 strains (33.33 %) were active against *S. Typhi,* and 9 strains (60 %) were active against *S. dysenteriae* respectively. From the Figure 3 result, 6 strains (40.00 %) were active against *S. aureus* P1, 8 strains (53.33 %) were active against *Vibrio* sp. MF2, 5 strains (33.33 %) were active against *Salmonella* sp. and 5 strains (33.33 %) were active against *Vibrio* sp. S1 respectively. Among the 15 isolates, 14 (93.33 %) strains had antibacterial activity while 1 (6.67 %) strain had no activity against human bacterial pathogens respectively. Also, among the 15 isolates 10 (66.67 %) strains had antibacterial activity while 5 (33.33 %) strains had no activities against fish and shell fish bacterial pathogens respectively. From the primary screening, strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 were found to be the two most potent strains out the 15 isolates to have high activities against human, fish and shell fish bacterial pathogens and hence selected for secondary screening based on their efficiencies. From the Figure 2 result, the crude chloroform extract of strain *S. lavendulae* ATS-3 exhibited prominent

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activity with large area of zone of inhibition against all the human bacterial pathogens used, having maximum significant (P < 0.05) inhibition of 30.00 ± 0.25 mm against *S. dysenteriae* and least significant (P < 0.05) inhibition of 10.00 ± 0.16 mm against *S. aureus* in comparison to the positive control ciprofloxacin inhibition of 20.00 ± 0.18 mm respectively. From the Figure 4 result, the crude chloroform extract of strain *S. lavendulae* ATS-1 also exhibited prominent activity with large area of zone of inhibition against all the fish and shell fish bacterial pathogens used, having maximum significant (P < 0.05) inhibition of 25.00 ± 5.20 mm against *Vibrio* sp. S1 and least significant (P < 0.05) inhibition of 11.00 ± 2.24 mm against *S. aureus* P1 in comparison to the positive control ciprofloxacin inhibition of 35.00 ± 5.15 mm respectively.

5. Determination of protein profile and taxonomic correlation

The result of the SDS-polyacrylamide gel electrophoresis of the partially purified whole cell proteins of strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 is shown in Plate 1 while Figure 5 shows the taxonomic relationship of the proteins sequences of strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 using hierarchal cluster algorithm method. From the Plate 3 result, SDS–PAGE of the protein preparation revealed double distinctive and prominent protein bands for the pure preparations of the partially purified whole cell proteins with an apparent lower and upper molecular weights of 71.7 kDa and 47.5 kDa and 70.4 kDa and 41.16 kDa for strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 respectively. Also, from the Figure 5 result, the hierarchal cluster algorithm analysis revealed that the protein sequences of the two strains are closely related to each other with 80 % similarities in the cluster dendogram.

RESULTS:

Table 1. Distribution of Actinomycetes in marine water and sediment

Sample	Dilution	Average number of colonies	Log CFU / ml /g
Marine water	10^{-1}	124	4.09 ± 0.05
Marine water	10^{-2}	53	4.72 ± 0.06
Marine sediment	10^{-1}	71	3.85 ± 0.08
Marine sediment	10^{-2}	53	4.72 ± 0.10

Table 2. Cultural characteristics of Actinomycetes isolates on tryptone yeast extract agar (ISP 1)

Isolates	Aerial mycelium	Substrate mycelium
ATS-1	Grayish green	Brown
ATS-2	Grayish	Brown
ATS-3	Whitish gray	Dark brown
ATS-4	Grayish green	Brown
ATS-5	Grayish green	Brown
ATS-6	Grayish green	Whitish brown
ATS-7	Grayish green	Yellow
ATS-8	Grayish green	Grayish yellow
ATS-9	Gray	Milky brown
ATS-10	Grayish green	Brown
ATS-11	Brownish white	Yellow
ATS-12	Light purple	Dark brown
ATS-13	Pink	Yellowish pink
ATS-14	Grayish green	Yellowish brown
ATS-15	Grayish green	Yellowish brown

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Table 3. Cultural characteristics of Actinomycetes isolates on yeast extract malt extract agar (ISP 2)

Isolates	Aerial mycelium	Substrate mycelium
ATS-1	Greyish yellow	Pinkish brown
ATS-2	Light gray	Pinkish brown
ATS-3	Yellowish green	Dark brown
ATS-4	Greyish yellow	Pink
ATS-5	Greyish green	Light brown
ATS-6	Greyish green	Yellow
ATS-7	Yellowish green	Pinkish brown
ATS-8	Yellowish green	Yellowish brown
ATS-9	Yellowish brown	Yellowish brown
ATS-10	Greenish yellow	Pinkish brown
ATS-11	Yellowish white	Yellow brown
ATS-12	Light purple	Yellowish brown
ATS-13	Yellowish green	Pinkish brown
ATS-14	Greyish green	Pinkish brown
ATS-15	Yellowish green	Black

Table 4. Cultural characteristics of Actinomycetes isolates on oatmeal agar (ISP 3)

Isolates	Aerial mycelium	Substrate mycelium
ATS-1	Grayish yellow	Pinkish brown
ATS-2	Yellowish green	Brown
ATS-3	Yellowish green	Brown
ATS-4l	Grayish yellow	Brown
ATS-5	Yellowish green	Yellowish brown
ATS-6	Yellowish green	Yellowish brown
ATS-7	Yellowish green	Brown
ATS-8	Yellowish green	Brown
ATS-9	Yellowish pink	Milky
ATS-10	Grayish yellow	Pinkish brown
ATS-11	Yellowish green	Milky
ATS-12	Yellowish green	Milky
ATS-13	Yellowish brown	Brown
ATS-14	Grayish yellow	Brown
ATS-15	Grayish yellow	Brown

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Table 5. Cultural characteristics of Actinomycetes isolates on yeast extract peptone iron agar (ISP 6)

Isolates	Aerial mycelium	Substrate mycelium	Melanoid pigment
ATS-1	Grayish green	Dark brown	Absent
ATS-2	Grayish green	Dark brown	Absent
ATS-3	Grayish green	Yellow	Absent
ATS-4	Grayish green	Dark brown	Absent
ATS-5	Grayish green	Dark brown	Absent
ATS-6	Yellow	Dark brown	Absent
ATS-7	Grayish green	Dark brown	Absent
ATS-8	Grayish green	Dark brown	Absent
ATS-9	Grayish green	Dark brown	Absent
ATS-10	Grayish green	Dark brown	Absent
ATS-11	Grayish green	Dark brown	Absent
ATS-12	Grayish green	Dark brown	Absent
ATS-13	Grayish green	Dark brown	Present
ATS-14	Grayish green	Dark brown	Absent
ATS-15	Grayish green	Dark brown	Present

Table 6. Biochemical and morphological characterization of marine Actinomycetes isolates

Iso	Ind	Ure	Glu	Ma	Lac	Sac	Ma	Sal	Xyl	Arc	Gel	Esc	Gly	Cel	Mn	Mlz	Raf	Sor	Rhc	Tre	Cat	Gr	Coc	Sp	Af	SSA
1	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+	+	-	-	S
2	-	+	+	-	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	S
3	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	R
4	-	-	+	-	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	S
5	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	R
6	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	S
7	-	+	+	-	-	+	+	-	+	-	+	-	-	+	+	+	-	-	-	+	-/+	+	-	-	-	RF
8	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-/+	+	-	-	-	RF
9	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	-	-	S
10	-	+	+	-	-	+	+	-	+	-	+	-	-	+	+	+	-	-	-	+	+	+	+	-	-	S
11	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	R
12	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	S
13	-	-	+	-	-	+	+	+	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	-	-	S
14	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	R
15	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	S

[-] = Absent, [+] = Present, ISO = Isolates, IND = Indole, URE = Urea, Glu= Glucose, MAN = Mannitol, LAC = Lactose, SAC = Sucrose, MAL = Maltose, SAL = Salicin, XYL = Xylose, ARA = Arabinose, GEL = Gelatin, ESC = Esculin, GLY = Glycerol, CEL = Cellobiose, MNE = Mannose, MLZ = Melezitose, RAF = Raffinose, SOR = Sorbitol, RHA = Rhamnose, TRE = Trehalose, CAT = Catalase, GR = Gram reaction, COC = Cocci (Morphology), SP = Endospore, AF = Acid fast, SSA= Spore structural arrangement, S = Spiral, R = Reticulum, RF = Rectiflexible.

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Table 7. Identification of the Actinomycetes isolates

Isolates	Genus	Species
ATS-1	*Streptomyces*	*lavendulae*
ATS-2	*Streptomyces*	*purpureus*
ATS-3	*Streptomyces*	*lavendulae*
ATS-4	*Streptomyces*	*purpureus*
ATS-5	*Streptomyces*	*lavendulae*
ATS-6	*Streptomyces*	*lavendulae*
ATS-7	*Actinomyces*	*viscosus* 1
ATS-8	*Actinomyces*	*viscosus* 2
ATS-9	*Streptomyces*	*exfoliates*
ATS-10	*Streptomyces*	*purpureus*
ATS-11	*Streptomyces*	*exfoliates*
ATS-12	*Streptomyces*	*purpureus*
ATS-13	*Streptomyces*	*cyaneus*
ATS-14	*Streptomyces*	*griseoviridis*
ATS-15	*Streptomyces*	*lavendulae*

Table 8. Biochemical characterization of fish and shell fish bacterial pathogens

Bacterial isolate	Oxidase test	Citrate test	Coagulase test	Hydrogen sulphide production
S. aureus P1	-	-	+	-
Vibrio sp. MF2	+	+	-	-
Salmonella sp.	-	+	-	+
Vibrio sp. S1	+	+	-	-

+ = present, - = absent

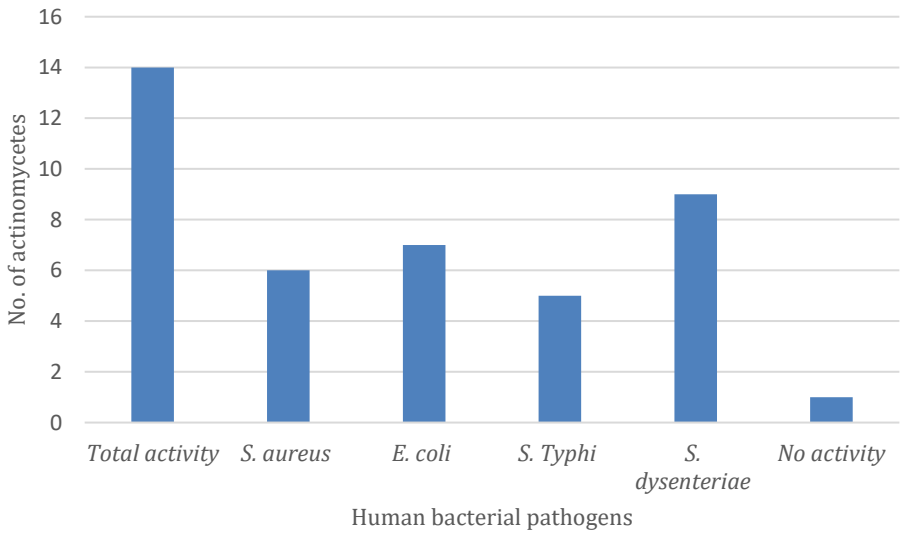

Fig. 1. Antibacterial activity of marine actinomycetes isolates against human bacterial pathogens

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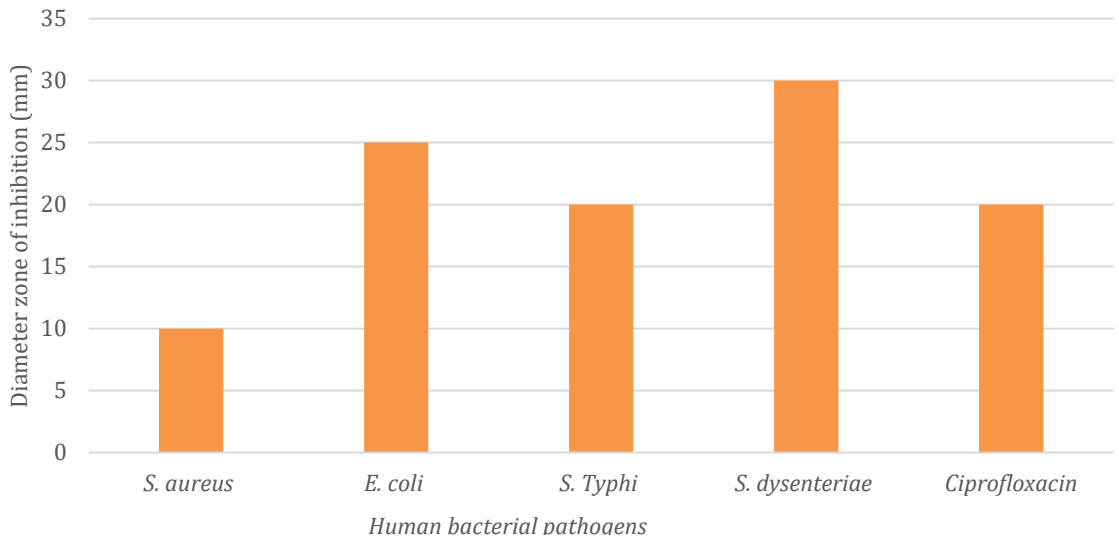

Fig. 2. Secondary metabolic activity of *Streptomyces lavendulae* ATS-3 against human bacterial pathogens

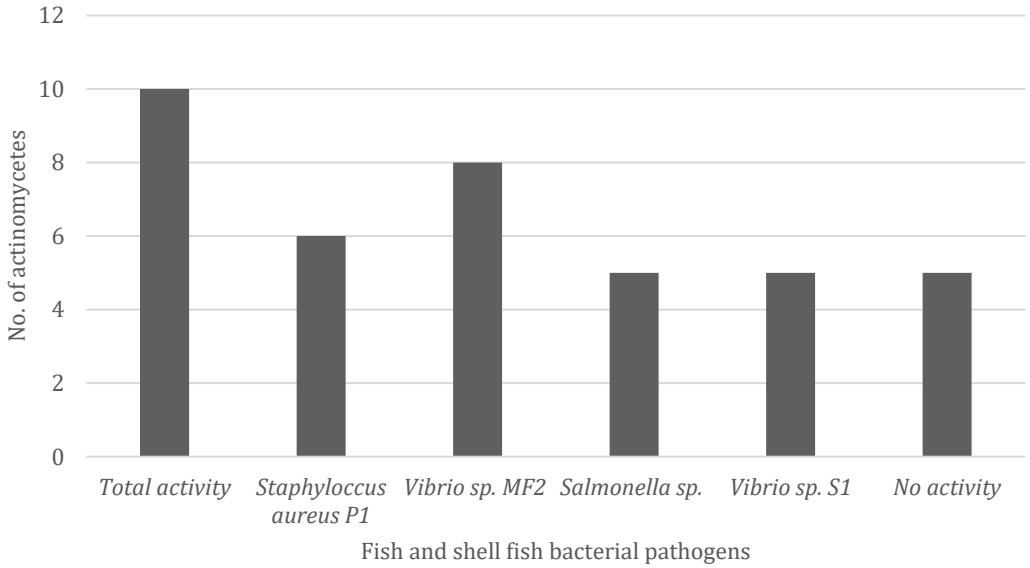

Fig. 3. Antibacterial activity of marine actinomycetes isolates against fish and shell fish bacterial pathogens

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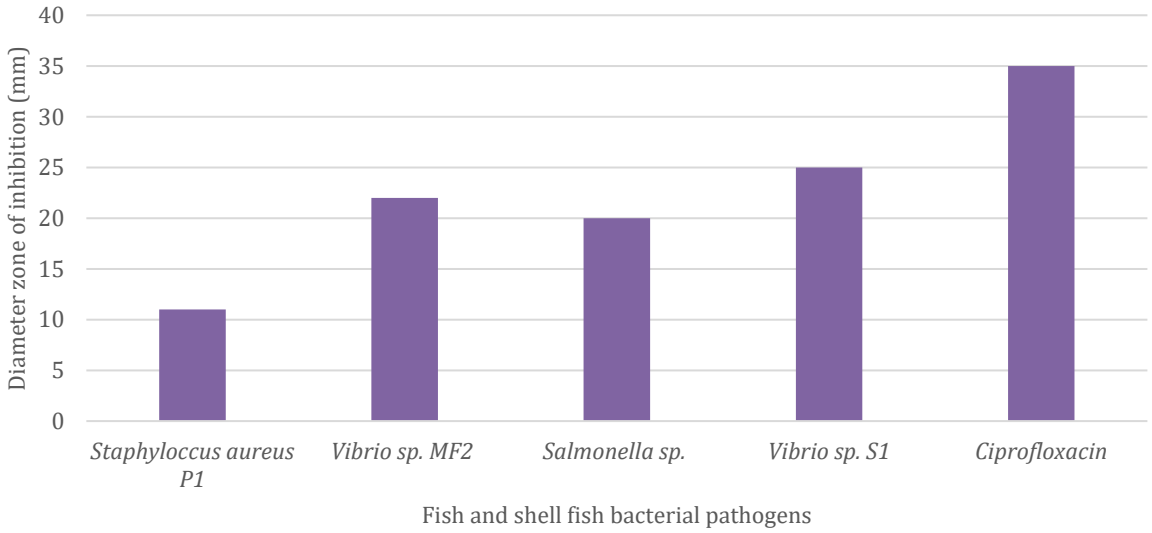

Fig. 4. Secondary metabolic activity of *Streptomyces lavendulae* ATS-1 against fish and shell fish bacterial pathogens

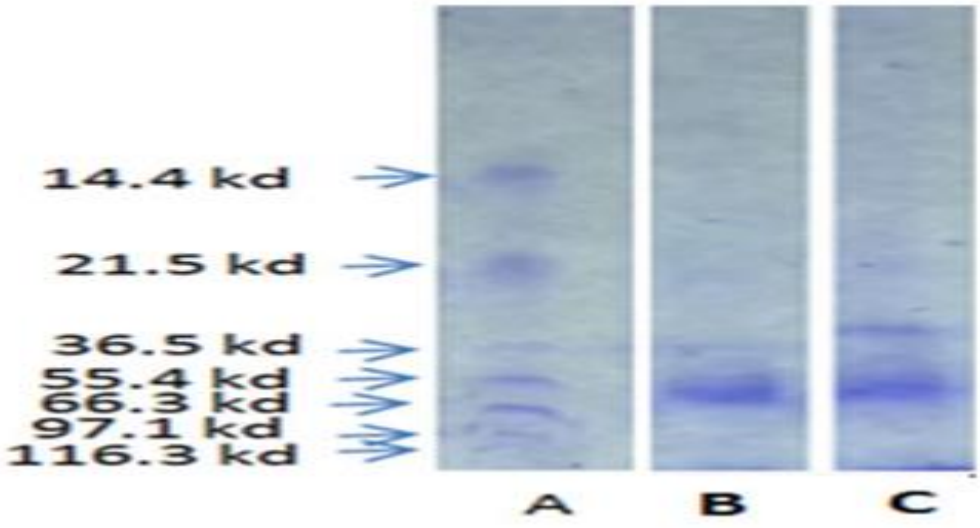

Plate 1. SDS-polyacrylamide gel electrophoresis of the partially purified whole cell proteins of strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1

DISCUSSION:

Actinomycetes comprise 10 % of the total bacteria colonizing marine aggregates. Marine habitat has been proven as an outstanding and fascinating resource for innovative potent bioactive producing microorganisms. Members of the Actinomycetes, which live in marine environment, are poorly understood and only few reports are available. Actinomycetes account for 70 % of the earth's surface and represent attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites [30]. In this study, an attempt was made to screen for the antibacterial activities and protein sequences of Actinomycetes isolated from Coastal Area of Niger Delta against human, fish and shell fish bacterial pathogens

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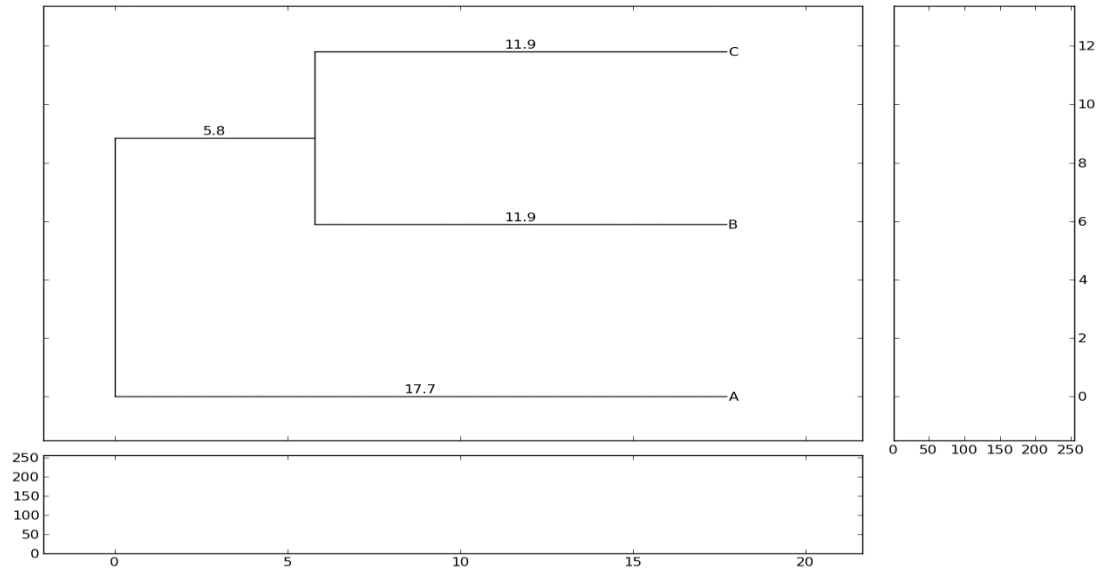

Fig. 5. Taxonomic relationship of the proteins sequences of strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 using hierarchal cluster algorithm method

The primary isolation of Actinomycetes was carried out on the selective media- Modified Glycerol Starch Casein Nitrate Agar supplemented with cycloheximide and nalidixic acid to prevent fungal and other bacterial contaminations. This same method was previously used by several researchers [1, 14, 16, 31, 32]. The result in Table 1 showed that the marine water was found to be richer in microbial count (4.09 ± 0.05 logCFU /ml) than the marine sediment microbial count (3.85 ± 0.08 logCFU /ml) indicating that surface water inhabits Actinomycetes more than the sediment. It also showed that bacterial colonies in both samples were minimal in number which could be as a result of the glycerol content of the selective media which inhibits the growth of bacteria [33]. Sekhar *et al.* [24] reported that among the 28 marine sediment samples, the total heterotrophic bacterial load ranged from 1.7 x 10^6 to 4.5 x 10^6 CFU /g of sediment.

The cultural characteristics of the fifteen isolates were studied using different International Streptomyces Project (ISP) media and were presented in Tables 2 to 5. The fifteen isolates showed varying colourations in their aerial and substrate mycelia. Melanoid pigment was synthesized by two of the isolates ATS 13 (*Streptomyces cyaneus*) and ATS 15 (*Streptomyces lavendulae*). Many authors have reported that most of the *Streptomyces*-like strains show grey aerial mycelia [1, 13, 14, 16, 26, 34]. The differences in the colour characteristics of the aerial mycelia of the isolates and the soluble pigments they produce, is an indication of the diversity of *Streptomyces* isolated from the sampling sites. These differences may be due to other primary as well as secondary metabolites from the enriched media [35].

All the strains were Gram positive, acid fast negative and endospore negative which is similar to the findings of Valli *et al.* [36]. The carbon utilization characteristics of the strains were studied and the result presented in Table 6. All the strains were indole negative and utilized glucose. Almost all the strains did not utilize mannitol except ATS-14 (*Streptomyces griseoviridis),* while only strain ATS-5 (*Streptomyces lavendulae)* out of the 15 isolates, was able to utilize lactose. The strains were not able to utilize gelatin except strains ATS-13 (*Streptomyces cyaneus)* and ATS-14 (*Streptomyces griseoviridis).* However, 20 % of the isolates were able to metabolize gelatin

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and urea respectively and 93 % isolates catabolized mannose (Table 6). All were cocci in shape except strains ATS-7 and ATS-8. Most were spirally arranged in spore chain followed by reticulum and rectiflexible simple aperture type of spore morphology and this correlates with the findings of Baskaran *et al.* [14] and Poorsarla *et al.* [26] who reported that their Actinomycetes isolates had spiral and rectiflexible shaped spore chains. The strains were identified using the API identification system software. The predominant genus belonged to *Streptomyces* and the least belonged to the *Actinomyces* (Table 7). Several previous reports from different geographical locations around the world have described the occurrences of *Streptomyces* in different mangrove habitats [10]. *Streptomyces* is known to be the most common genus in the marine environment especially in the shallow organic rich coastal areas [37]. Several streptomycetes, *viz.*, *Streptomyces alboniger*, *Streptomyces violaceus, Streptomyces moderatus and Streptomyces aureofasciculus* were also reported from the Vellar estuary on the Southeast coast of India [15]. The Actinomycetes classification system was mainly dependent on characteristics like the form of spores and use of carbon. The nutritional uptake, physiological and biochemical characteristics were clearly proved under the classification [38].

The result in Table 8 showed that *Vibrio* sp. MF2 and *Vibrio* sp. S1 were positive to oxidase test, *Salmonella* sp. and *Vibrio* sp. were positive to citrate test, *S. aureus* P1 was positive to coagulase test while *Salmonella* sp. was positive to hydrogen sulphide production [6, 8, 22].

The Actinomycetes are noteworthy antibiotic producers, making the quarters of all known pharmaceutical products; the streptomycetes are especially prolific [14]. In this study, the result in Figure 1 revealed that among the 15 isolates, 14 (93.33 %) strains had antibacterial activity while 1 (6.67 %) strain had no activity against human bacterial pathogens. Also, the result in Figure 3 also revealed that among the 15 isolates, 10 (66.67 %) strains had antibacterial activity while 5 (33.33 %) strains had no activities against fish and shell fish bacterial pathogens respectively from the primary screening. Baskaran *et al.* [14] reported that out of 42 Actinomycetes from the mangrove sediments of Andaman and Nicobar Islands, 22 (58.4%) isolates had antibacterial activity against pathogenic bacteria namely *B. subtilis, S. Typhi, S. aureus* and *K. pneumoniae.* All the 42 isolates showed antibacterial activity with at least one test bacteria. Sunaryanto and Marwoto [39] reported that eight of the 29 Actinomycetes isolates showed antibacterial activity, 2 isolates active against *E. coli* ATCC 25922, 4 isolates active against *S. aureus* ATCC25923, 2 isolates active against *B. subtilis* ATCC 66923 and 3 isolates active against *P. aeruginosa* ATCC27853. Dharmaraj [22] reported that the initial screening of the isolates exhibited antibacterial activity against fish and shell fish bacterial pathogen *Aeromonas hydrophila.* From the primary screening, strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 had significant antibacterial activities out of the 15 isolates against human, fish and shell fish bacterial pathogens and hence selected for secondary screening based on their efficiencies. The result in Figure 2 revealed that the crude chloroform extract of strain *S. lavendulae* ATS-3 exhibited maximum significant ($P < 0.05$) inhibition of 30.00 ± 0.25 mm against *S. dysenteriae* and least significant ($P < 0.05$) inhibition of 10.00 ± 0.16 mm against *S. aureus* in comparison to the positive control ciprofloxacin inhibition of 20.00 ± 0.18 mm respectively. The result in Figure 2 also revealed that the crude chloroform extract of strain *S. lavendulae* ATS-1 also exhibited maximum significant ($P < 0.05$) inhibition of 25.00 ± 5.20 mm against *Vibrio* sp. S1 and least significant ($P < 0.05$) inhibition of 11.00 ± 2.24 mm against *S. aureus* P1 in comparison to the positive control ciprofloxacin inhibition of 35.00 ± 5.15 mm respectively. The results indicated that the crude chloroform extracts of the most potent strains possessed antibacterial

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potentials but lower in activity than the standard antibiotic ciprofloxacin (CPX) against fish and shell fish bacterial pathogen. These findings imply that the antibacterial compound was related to the type of solvent used for extraction and the particular group of pathogens acted against. Rosmine and Varghese [10] reported that the methanol extract of strain ER7 was found to contain the antimicrobial compound as it was active against all the human and fish pathogens tested. Pushpa and Doss reported that out of the four elutes of purified proteins of marine Actinomycetes, elute 1 showed activity against all human pathogens with growth inhibition against *Klebsiella pneumoniae* (8 mm), *Proteus vulgaris* (8 mm), *Salmonella Typhi* (8 mm), *Streptococcus pyogenes* (7 mm) and *Pseudomonas aeruginosa* (7 mm) while elute 1 also showed the activity against all fish pathogens such as *Aeromonas hydrophila* (9 mm), *Vibrio alginaticus* (9 mm), *Bacillus subtilis* (8 mm), *Pseudomonas aeruginosa* (8 mm) and *Vibrio harveyi* (7 mm). The present study also revealed the two potent strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 had significant antibacterial activities against Gram negative more than the Gram positive tested pathogens. The present result contradicts the findings of Kokare *et al.* [40], Devi *et al.* [41] and Valli *et al.* [36] who reported that Actinomycetes often encountered in marine ecosystem showed more active antimicrobial activity against Gram positive bacteria than Gram negative bacteria. Dhanasekaran *et al.* [42] found that estuarine Actinomycetes which remained largely ignored showed promising antibacterial activities. There are some reports of actinobacterial strains from marine sediments being used against shrimp pathogens like *Vibrio* spp. [22, 43].

The protein profiling was done to identify differentiation among the strains that exhibited bioactivity [22]. Bacterial whole-cell protein profiles can be analyzed by polyacrylamide gel electrophoresis to generate complex banding patterns that can be used to delimit species and subspecies groups. Proteins can be analyzed either by using 1-dimensional (1-D) or 2-dimensional protein electrophoresis. Many bacterial strains can be compared rapidly and reproducibly with 1-D protein electrophoresis [44]. The result in Plate 1 showed that the double distinctive and prominent protein bands of the partially purified bacterial whole cell proteins of the two potent strains *S. lavendulae* ATS-3 and *S. lavendulae* ATS-1 were electrophoretically homogenous. This was confirmed in the taxonomic cluster dendogram which revealed that the protein sequences of the two strains were closely related to each other with 80 % similarities (Figure 5) suggesting that the actinobacterial strains belonged to the genus *Streptomyces*. Previous investigations used SDS-PAGE to analyze and compare the cellular protein profiles of 32 *Streptomyces* strains and 5 *Streptoverticillium* strains [45]; 7 *Streptomyces* strains [22] and 6 Actinomycetes isolates [28]. Taxonomic correlations were found between the profiles obtained and the phenotypic groupings observed earlier [46].

CONCLUSION:

The study clearly revealed that Onne light terminal flow is a potent source for the isolation of bioactive Actinomycetes. The screened Actinomycetes strains especially strains *S. lavendulae* (ATS-3) and *S. lavendulae* (ATS-1) were also found morphologically distinct with significant ($P < 0.05$) potent antibacterial activity against human, fish and shell fish bacterial pathogens and hence possessing promising potentials to be used as potent source of novel antibiotics and probiotics especially in aquaculture. Further purification of the expended medium could give more activity than the standard antibiotics and also effective against some multidrug resistant bacterial pathogens.

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CONFLICTS OF INTERESTS:

Authors have declared that there is no conflict of interest as regarding the publication.

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